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Elicitor-Induced Ethylene Biosynthesis in Tomato Cells

Characterization and Use as a Bioassay for Elicitor Action

Georg Felix, Debora G. Grosskopf, Martin Regenass, Christoph W. Basse, and Thomas Boller*
Friedrich Miescher-Institute, P.O. Box 2543, CH-4002 Basel, Switzerland

ABSTRACT

The induction of ethylene biosynthesis by an elicitor partially purified from yeast extract was studied in suspension-cultured tomato (*Lycopersicon esculentum* Mill.) cells. Unstimulated cells produced little ethylene during exponential growth and even less in stationary phase. Treatment with elicitor stimulated ethylene biosynthesis 10-fold to 20-fold in the exponentially growing cells and more than 100-fold in stationary cells. Activities of both 1-aminocyclopropane-1-carboxylate (ACC) synthase, measured *in vitro*, and ethylene-forming enzyme (EFE), measured *in vivo*, increased strongly in response to elicitor treatments. During exponential growth, cells contained large pools of ACC, and the elicitor stimulated ethylene biosynthesis primarily through induction of EFE. In the stationary phase, cells contained almost no ACC, and the elicitor stimulated ethylene biosynthesis primarily through its effect on ACC synthase activity. Cordycepin did not affect the increase in activity of ACC synthase but blocked that of EFE, indicating that the former was posttranscriptionally regulated, the latter transcriptionally regulated. Removal of elicitor by washing or inactivation of a biotinylated derivative of the elicitor by complexation with avidin caused a rapid cessation of the increase in ACC synthase activity, suggesting that continuous presence of stimulus is necessary for the response. Using induction of ethylene production to measure amounts of elicitor, it was found that the elicitor disappeared from the incubation medium during the course of the treatment.

The reaction of suspension-cultured plant cells to fungal cell wall preparations, so-called elicitors, has been studied extensively as a model of the plant's defense response (2, 11, 15). This model is based on the premise that plant cells use elicitors as chemical cues to sense a pathogen attack and to react appropriately. Although the existence and identity of elicitors remain obscure in most plant-pathogen interactions (2, 11, 15), suspension-cultured plant cells have indeed an acute chemosensory perception system for a variety of elicitors and react to them with the transcriptional activation of a number of genes, including those coding for enzymes of the phenylpropanoid pathway (11, 15). Some of these genes appear to be activated within 5 min (13).

An early reaction of plant cells to elicitors is an increase in ethylene biosynthesis (2, 3, 9, 17, 18, 20, 22, 23). We found earlier that an increase in activity of ACC[1] synthase, the key enzyme in ethylene biosynthesis (25), is a particularly rapid response to an elicitor treatment in suspension-cultured parsley cells (5), noting that the induction of this enzyme probably occurred at the post-transcriptional level since it was not inhibited by cordycepin.

In the present study, we have characterized ethylene biosynthesis in suspension-cultured tomato cells in order to use it as a bioassay for the elicitor response. Our results show that elicitors cause rapid stimulation of ethylene biosynthesis and large increases in the activities of both enzymes involved in its biosynthesis (25), ACC synthase and EFE, throughout the culture cycle. Induction of the enzymes occurred with different kinetics and showed differential sensitivity to inhibitors of transcription and translation. Using induction of ethylene production as a bioassay, we demonstrate that elicitor activity rapidly disappears from the culture medium in the presence of plant cells. We further show that withdrawal of elicitors, either by washing or by addition of avidin to a biotinylated derivative of the elicitor, stops the increase in ACC synthase activity within 20 to 30 min. Thus, the level of ACC synthase remains elevated only as long as the cells are exposed to elicitor, and therefore closely monitors the actual state of elicitor perception.

MATERIALS AND METHODS

Cell Culture

A suspension culture was prepared from a callus derived from a Msk8 plant (kindly provided by M. Koornneef, Wageningen, The Netherlands), a sibling of a hybrid between *Lycopersicon esculentum* Mill. and *Lycopersicon peruvianum* Mill. backcrossed three times to *L. esculentum* (14), and cultivated at 25°C in a Murashige-Skoog type liquid medium supplemented with 5 μM 1-naphthylacetic acid, 1 μM 6-benzyladenine, and vitamins as described by Adams and Townsend (1). Cells were subcultured in intervals of 3 weeks by placing 1.5 to 3.0 g cells (fresh weight) into 50 mL of filter-sterilized fresh medium in autoclaved 250-mL flasks (Bellco International, Feltham, England) with aluminum caps.

Elicitor Preparations

A glycopeptide elicitor preparation called YE was prepared according to an extension of the protocol of Hahn and Albersheim (12), as will be described in detail elsewhere (CW Basse and T Boller, manuscript in preparation). Briefly, the fraction

[1] Abbreviations: ACC, 1-aminocyclopropane-1-carboxylic acid; EFE, ethylene forming enzyme; YE, yeast extract-derived elicitor.

of autolyzed yeast extract (Difco, Detroit, MI) soluble in 60% (v/v) ethanol but insoluble in 80% (v/v) ethanol was dialyzed and chromatographed on a DEAE-Trisacryl column (IBF Biotechnics, Villeneuve-la-Garenne, France). The nonadsorbed fraction was adsorbed at pH 6.0 on a SP-Trisacryl column and eluted with 500 mM NaCl. This preparation was bound to Con A agarose (Sigma Chemical Co., St. Louis, MO), eluted with 0.2 M α-methyl mannoside, dialyzed, and weighed.

N-Biotinylation of YE was performed by incubation with an excess of biotin-*N*-hydroxysuccinimide (Fluka, Buchs, Switzerland) by the procedure of Finn and Hofmann (8). After dialysis, biotinylated compounds were affinity-purified on a mono-avidin Sepharose column (Sigma) as described by the manufacturer.

Xylanase was obtained from Fluka, and the partial acid hydrolysate of cell walls of *Phytophthora megasperma* forma specialis *glycinea* was a kind gift of Dr. J. Ebel (Institut für Biologie 2, Universität Freiburg i. Br., FRG).

Analytical Methods

The rate of ethylene production was determined as follows. Portions (2 mL) of the suspension culture containing 0.2 to 0.4 g cells (fresh weight) were enclosed with 1.5 to 3 mL of air in 5-mL plastic syringes (Becton Dickinson, Dublin, Ireland) sealed at the tip with rubber caps. The syringes were placed horizontally on a rotary shaker and shaken at 100 to 150 rotations/min to allow aeration. In intervals of 30 min, the air phase (1 mL) was analyzed for ethylene on a gas chromatograph equipped with an aluminum oxide column and a flame-ionization detector. Identification and quantification were carried out by comparison with the retention time and peak area of standard samples. After each interval, the air was replaced with fresh air. The rate of ethylene production (nmol h^{-1} g^{-1} fresh weight) was calculated for each interval and plotted at its endpoint.

Activity of EFE was determined *in vivo* by supplementing cell suspension cultures with 0.1 mM ACC and measuring ethylene production as described above. (It should be noted that ethylene production was only slightly stimulated by the exogenous ACC in exponentially growing cells, due to their relatively high content in endogenous ACC. ACC was added simply to saturate EFE activity.)

Activity of ACC synthase was measured in permeabilized cells as described before (20), except in the experiment illustrated in Figure 3 where it was measured in cell extracts as described previously (5).

The level of ACC in tissue extracts was determined according to the method of Lizada and Yang (16), and the protein content was measured according to Bradford (4).

RESULTS

Induction of Ethylene Biosynthesis in Response to Fungal Elicitors

Tomato cells of line Msk8 were treated with various elicitor preparations known to induce ethylene biosynthesis and defense reactions in other systems (5, 9, 10). Several different elicitor preparations stimulated ethylene biosynthesis in a concentration-dependent manner (Fig. 1). A crude acid hydrolysate from cell walls of *P. megasperma* forma specialis *glycinea* induced ethylene biosynthesis maximally at concentrations of 10 to 30 μg mL^{-1}. Commercial xylanase caused considerable stimulation of ethylene biosynthesis at concentrations of 0.1 μg mL^{-1} and induced ethylene biosynthesis maximally at 10 μg mL^{-1}. A partially purified elicitor derived from YE caused significant increases of ethylene biosynthesis at concentrations of 0.3 μg mL^{-1}. Stimulation of ethylene biosynthesis increased continually for YE concentrations between 0.1 and 100 μg mL^{-1} (Fig. 1). YE was chosen to study elicitor-induced ethylene biosynthesis in more detail. As a basis for this, biosynthesis of ethylene was first studied in unstimulated cells.

Ethylene Biosynthesis, ACC Level, and Activity of the Ethylene Forming Enzyme during a Culture Cycle

Tomato cells started to accumulate proteins at an exponential rate soon after subculture and reached a plateau of protein content after about 6 d, whereas their fresh weight started to increase only about 2 d after subculture and reached a plateau after about 10 d at a fresh weight of about 30 g/50 mL (Fig. 2A). The rate of ethylene biosynthesis, determined by sealing the flasks for 1 h, varied considerably during the culture period. It increased from values below 10 pmol g^{-1} h^{-1} to 200 pmol g^{-1} h^{-1} during the first 3 to 4 d of subculture and decreased thereafter, dropping to values below 10 pmol g^{-1} h^{-1} after 12 d (Fig. 2B). ACC accumulated in the cells in the early phase of the growth cycle and dropped to very low levels during the stationary phase (Fig. 2C). This was in accordance with the finding that addition of ACC had little effect on the rate of ethylene biosynthesis between 3 and 9 d after subcul-

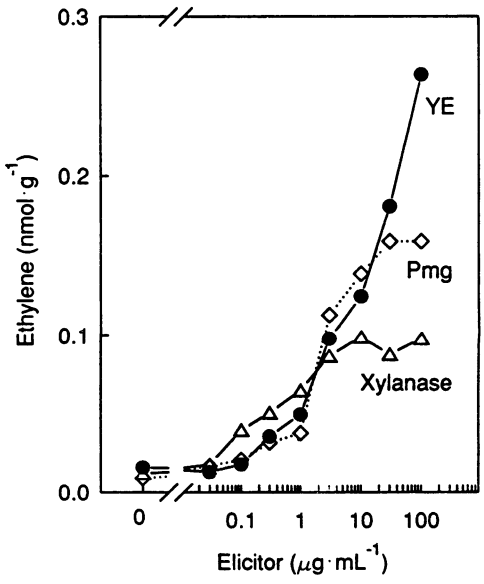

Figure 1. Stimulation of ethylene biosynthesis in tomato cells by various elicitors. Cells were treated in the stationary phase with different amounts of YE, commercial xylanase, or a partial acid hydrolysate from cell walls of *P. megasperma* (Pmg). Ethylene production was measured for the interval of 0 to 4 h after elicitation.

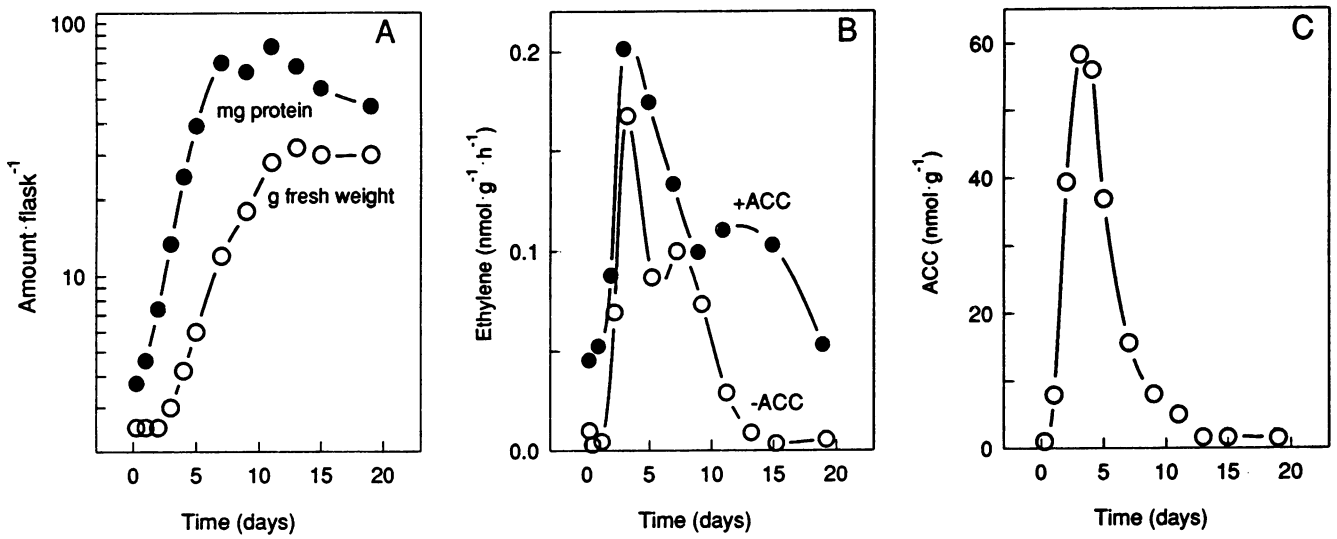

Figure 2. Growth of tomato cells (A), and their ethylene production (B) and ACC levels (C), at different stages of the culture cycle. At time zero, 2.5 g cells were inoculated per flask containing 50 mL fresh medium. Growth was followed as fresh weight (O) and protein content (●). In B, ethylene production rate was measured in the presence (●) or absence (O) of ACC (100 μM).

ture but increased up to 25-fold during stationary phase (Fig. 2B).

Induction of Ethylene Biosynthesis by Elicitors during a Culture Cycle

Induction of ethylene biosynthesis by YE was tested at different times after subculture by enclosing aliquots of the suspensions, adjusted to 0.4 g cells/2 mL by filtration and resuspension in their own medium, into 5-mL plastic syringes and measuring ethylene production in 30-min intervals for 4 h. Without addition of YE, the rates of ethylene production deduced from the last interval between 3.5 and 4.0 h (Fig. 3A) were similar to the rates in culture flasks (Fig. 2B; note difference in scale), indicating that shaking in plastic syringes did not affect ethylene biosynthesis. Treatment with elicitor for 4 h strongly induced the rates of ethylene production throughout the culture cycle (Fig. 3A). In absolute terms, the elicitor-stimulated rate of ethylene production reached a peak of approximately 2 nmol g^{-1} h^{-1} at the end of the exponential growth phase, 5 to 7 d after subculture (Fig. 3A). However, the strongest stimulation of ethylene biosynthesis by elicitor in relative terms, over 100-fold compared to untreated control cells, was reached only in the stationary phase, due to the low production rates of untreated control cells at this stage.

The question arose as to whether the induction of ethylene

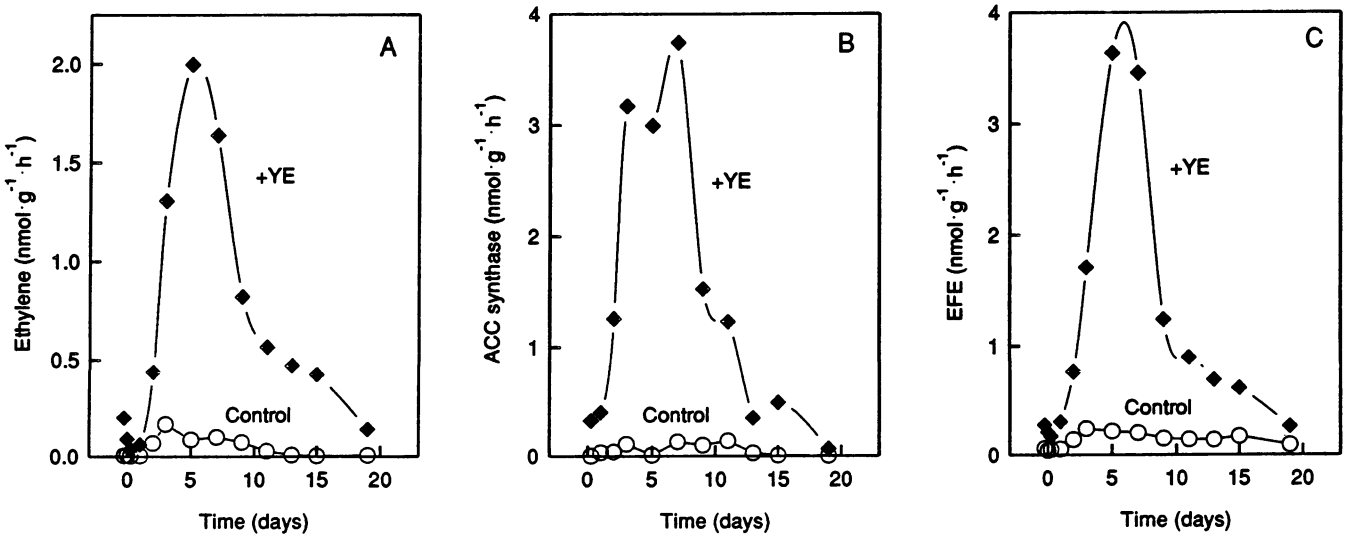

Figure 3. Effect of elicitor on ethylene biosynthesis (A), ACC synthase (B), and EFE (C) in tomato cells at different stages of a culture cycle. Plant cells were treated with 10 μg mL^{-1} YE. The rate of ethylene biosynthesis and EFE activity are given for the interval of 3.5 to 4 h after elicitation. ACC synthase activity was measured in cells treated with elicitor for 2 h.

biosynthesis observed was due to increases in ACC synthase, EFE, or both. ACC synthase activity was low in untreated cells throughout the culture cycle. While the activity could well be measured between 2 and 12 d of the culture cycle, it was at the detection limit during the early and late stage of the culture cycle. ACC synthase activity was induced rapidly and transiently upon treatment with YE (see Fig. 6A below) and increased at least 10-fold within 2 h throughout the culture cycle (Fig. 3B).

A similar but somewhat less rapid response was observed for EFE. Its activity also increased at least 10-fold in response to a 4-h treatment with YE throughout the culture cycle (Fig. 3C). Like elicitor-stimulated ethylene biosynthesis, elicitor-stimulated EFE activity reached its highest absolute values towards the end of the exponential growth phase, 5 to 7 d after subculture. The strongest stimulation of EFE in relative terms, about 15-fold compared with untreated control cells, was reached during the same culture period.

Time Course of Elicitor-Induced Ethylene Biosynthesis and Effects of Inhibitors of Transcription and Translation

Ethylene production started to increase rapidly after elicitor treatment. It doubled within 1 h and reached an approximate 25-fold stimulation within about 4 h (Fig. 4A). Cells had a low but measurable ACC synthase activity of 20 pmol g^{-1} h^{-1} at the onset of the treatment with YE. The activity increased almost 30-fold within 1 h and reached 3 nmol g^{-1} h^{-1} after 4 h (Fig. 4B). Activity of EFE had a comparatively high value of 200 pmol g^{-1} h^{-1} at the onset of treatment. It increased gradually after a lag of about 1 h, reaching 2.4 nmol g^{-1} h^{-1} after 4 h (Fig. 4C).

Simultaneous addition of cycloheximide with YE completely prevented induction of ethylene biosynthesis, ACC synthase, and EFE (data not shown). When cycloheximide (40 μM) was added 2.5 h after YE, it immediately blocked further increases of ethylene production, ACC synthase, and EFE activities (Fig. 4). As reported previously (20), activity of ACC synthase decayed rapidly, with a half-life of about 60 min, while EFE activity decreased with a half-life of about 4 h. The slow and constant decrease of EFE, which is highly sensitive to uncouplers and metabolic inhibitors (25), indicates that even the relatively high concentration of cycloheximide used had no unspecific side effects.

Chappell *et al.* (5) had previously reported that the elicitor-dependent increase of ACC synthase in parsley cells was not affected by cordycepin, an inhibitor of mRNA synthesis. The effect of this inhibitor was tested in tomato cells. Cordycepin (0.2 mM), given 2 h before YE, slowed the induction of ACC synthase to some extent but did not prevent it (Fig. 5A). However, it blocked induction of EFE nearly completely (Fig. 5B). As reported elsewhere (21), functional expression of mRNA for EFE in *Xenopus* oocytes directly demonstrated that the inhibitory effect of cordycepin on EFE induction was due to an inhibition of mRNA accumulation for EFE.

Concentration-Dependence of Induction of ACC Synthase by Elicitor as Related to Elicitor Disappearance

YE stimulated ethylene production in a concentration-dependent manner (Fig. 1). The time course of ACC synthase induction was compared for different concentrations of YE (Fig. 6A). YE at 0.1 μg mL^{-1} had no effect on ACC synthase activity. YE at 1, 10, and 100 μg mL^{-1} caused a rapid increase in ACC synthase, and significantly elevated levels were measured after only 10 min. While the increase continued at a similar rate during the first 40 to 60 min for all three concentrations, the activity reached its maximum and started to decrease at different times, *i.e.* after 60 min for 1 μg mL^{-1}, after 90 min for 10 μg mL^{-1}, and after 120 min for 100 μg mL^{-1} (Fig. 6A).

To explain this concentration-dependent duration of ACC synthase induction, we hypothesized that elicitor activity disappeared from the medium during incubation. To test this,

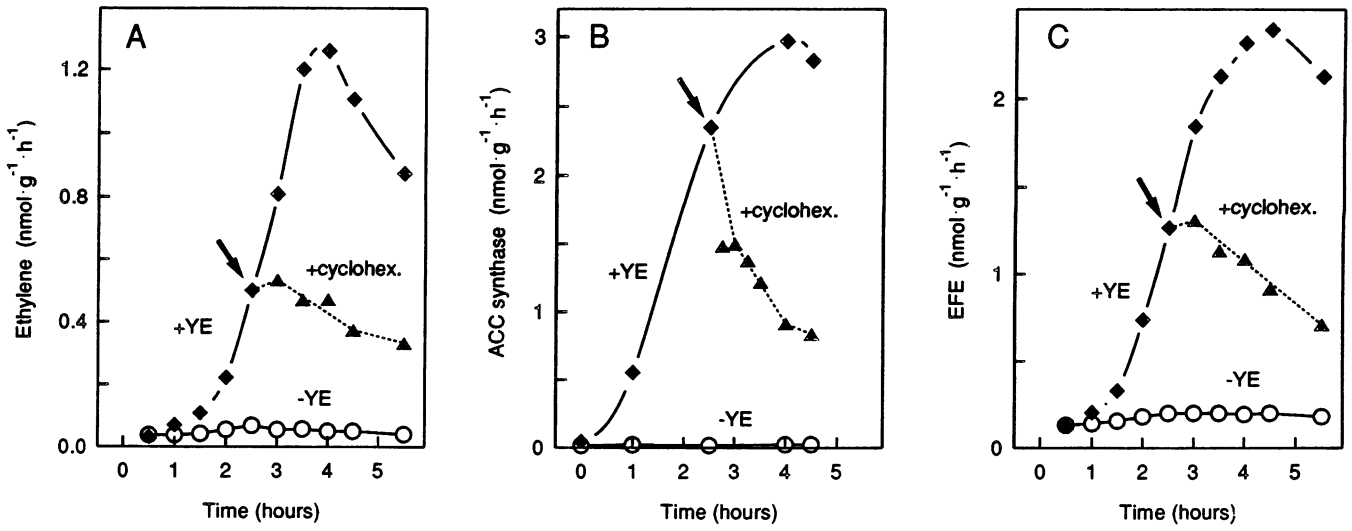

Figure 4. Effect of cycloheximide on the induction of ethylene biosynthesis (A), ACC synthase (B), and EFE (C) by elicitor. Cells (2 d after subculture) were treated with YE (10 μg mL^{-1}). Cycloheximide (40 μM) was added 2.5 h later.

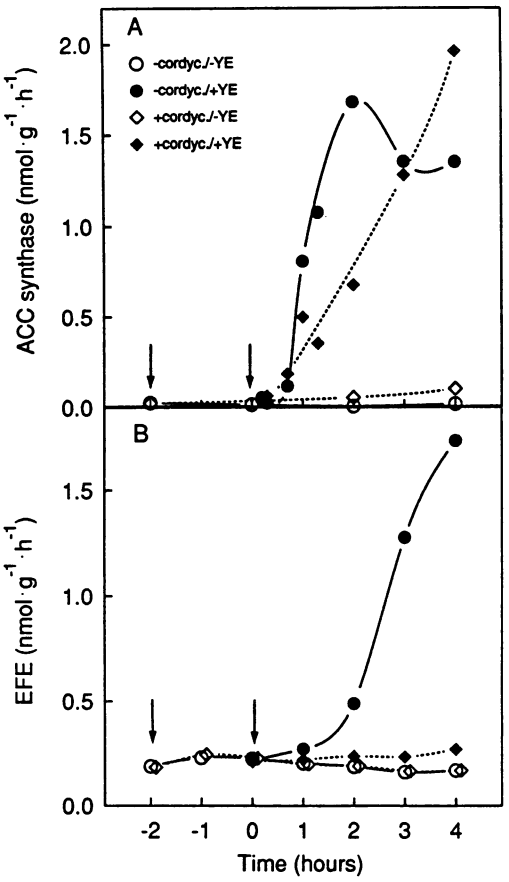

Figure 5. Effect of cordycepin on the induction of ACC synthase (A) and EFE (B). Cells (2 d after subculture) were preincubated with 0.2 mM cordycepin (◇, ◆) or without additions (○, ●) for 2 h before addition of 10 μg mL^{-1} YE (◆, ●) or an equivalent amount of water (◇, ○) at time zero.

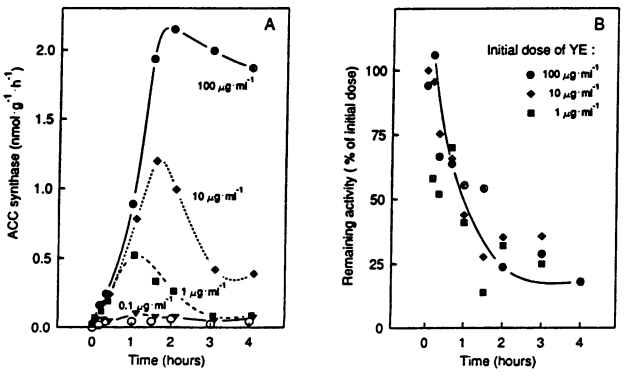

Figure 6. Time course of ACC synthase activity (A) and of elicitor concentration in the incubation medium (B) after addition of different amounts of YE. Elicitor levels in the medium were estimated by comparing their capacity to induce ethylene production to a dilution series of YE in a second batch of cells. In A, open circles represent values for untreated control cells.

aliquots of the media were harvested by filtration after different incubation times and tested for their capacity to induce ethylene biosynthesis in comparison to a dilution series of YE, using a second sample of cells (Fig. 6B). Elicitor activity indeed disappeared in a time-dependent manner. However, considerable elicitor activity remained in the medium even after 3 to 4 h, at a time when ACC synthase activity already started to decrease. Hence, the observed decrease of elicitor activity alone cannot explain the decrease of ACC synthase activity after 2 to 3 h.

Effect of Withdrawal of Elicitor on Activity of ACC Synthase

The data presented above suggest that continuous presence of elicitor is necessary to maintain the induction of ACC synthase. To test this more directly, the effect of withdrawal of elicitor was studied using two experimental approaches. In the first, cells washed and equilibrated in fresh growth medium were treated with YE for 30 min and then washed and resuspended in elicitor-free growth medium again (Fig. 7). ACC synthase activity continued to increase for only about 30 min after resuspension and then decreased abruptly, while control cells with elicitor showed a steady increase in ACC synthase (Fig. 7). Washing elicitor-treated cells and resuspending them in elicitor-containing fresh medium had little effect on the time course of ACC synthase induction (data not shown).

In the second experiment, cells were treated with biotinylated YE, an elicitor that strongly binds to avidin. To allow efficient complex formation between biotin and avidin, the incubation medium was buffered with 20 mM Pipes to pH

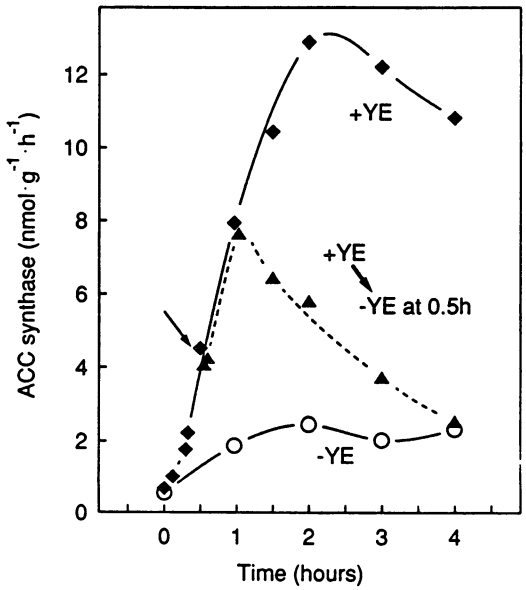

Figure 7. Effect of addition and removal of elicitor on the activity of ACC synthase. Cells (3 d after subculture) were harvested by filtration, washed, and resuspended in fresh medium. One hour later (*i.e.* at time zero), the suspensions were treated with 20 μg mL^{-1} YE elicitor (◆). A part of the culture was withdrawn after 30 min, washed, and resuspended in fresh medium without elicitor (▲).

6.8, a treatment that lowered inducibility of ACC synthase about twofold. When avidin was added together with the biotinylated YE at the beginning of the experiment, it completely prevented induction of ACC synthase (Fig. 8). The same concentration of avidin had no effect on induction of ACC synthase by normal, nonbiotinylated elicitor (data not shown). When avidin was given 40 min after the elicitor, induction of ACC synthase continued for approximately 20 min and then stopped (Fig. 8). The results of both experiments directly demonstrate that the continuous presence of elicitor is necessary for ACC synthase induction.

DISCUSSION

Induction of ethylene biosynthesis is an early reaction of plants to pathogens. Although the significance of enhanced ethylene biosynthesis in plant-pathogen interactions is not clear (2, 3, 17), it accompanies and, indeed, precedes many of the well-studied biochemical defense reactions such as induction of enzymes of the phenylpropanoid pathway and phytoalexin accumulation (11, 15). Having found that various elicitors induce ethylene biosynthesis and phenylalanine ammonia-lyase to a similar extent in tomato cells (10), we wanted to evaluate the use of ethylene production as a simple, easily measurable bioassay to monitor the elicitor response.

An important asset for a bioassay is its independence of changes in the physiological state of the cells. In the tomato cells chosen for our studies, induction of ethylene biosynthesis by elicitor can readily be measured throughout the culture cycle. Closer examination of the enzymatic basis for this induction, however, reveals changes during the culture cycle. During exponential growth, control cells have large pools of ACC and are limited in their ethylene production at the level of EFE. Therefore, induction of ACC synthase has almost no significance for the enhanced ethylene production. During stationary phase, the ACC pool disappears (mainly through conjugation to malonyl-ACC, data not shown), whereas a basal EFE activity remains. At this stage, induction of ethylene production initially reflects only the increase in ACC synthase and resembles the regulation of ethylene biosynthesis in pathogen-infected tomato leaves (19).

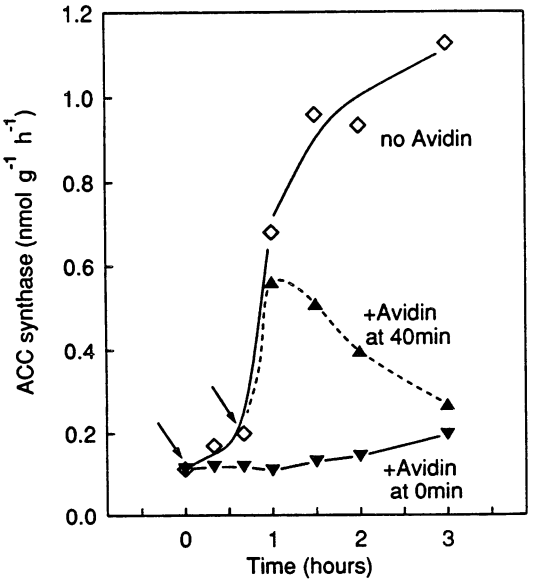

Figure 8. Effect of avidin on the induction of ethylene biosynthesis by a biotinylated elicitor. Cell suspensions (4 d after subculture) were supplemented with Pipes (Na^+), 20 mM final concentration, to buffer the pH value at pH 6.8. One hour later, biotinylated elicitor (approximately 10 μg mL^{-1}) was added at time zero, followed either immediately or 40 min later by avidin (200 μg mL^{-1}).

Most of the elicitor-induced enzymes examined so far are induced at the transcriptional level (11, 15). EFE appears to be a new member of this group of enzymes, since its induction is almost completely blocked by cordycepin. Functional expression of mRNA in *Xenopus* oocytes has been used to verify that cordycepin acts by inhibiting accumulation of mRNA for EFE (21). Interestingly, induction of ACC synthase by elicitors, although requiring *de novo* protein synthesis (Fig. 4B; ref. 5), is little affected by cordycepin (Fig. 5A; ref. 5) and therefore seems to occur at a posttranscriptional level. ACC synthase has recently been cloned from tomato fruits, and the steady-state levels of its mRNA have been shown to increase strongly upon wounding (24). It will be interesting to perform similar studies in elicitor-stimulated tomato cells.

ACC synthase is subject to rapid turnover, and its activity decreases rapidly after elicitor is withdrawn from the cells by washing or by adsorption of the biotinylated elicitor derivative to avidin (Figs. 7 and 8). These results show that the increase in ACC synthase activity requires continued presence of elicitor and that ACC synthase stimulation is closely tied to elicitor perception. In this regard, it is interesting that the increase in ACC synthase activity stops after about 2 or 3 h in cells stimulated with 10 or 100 μg mL^{-1} YE, respectively (Fig. 8A). Although disappearance of about 60% of the elicitor activity initially present can be monitored during this time, there remains sufficient activity in the incubation medium to stimulate previously untreated cells even 4 h after beginning of the treatment (Fig. 8B). The decay of ACC synthase may be a consequence of chemosensory adaptation, an aspect deserving a more thorough study.

Inspection of Figure 6A calls attention to difficulties in interpretation of data on elicitor responses in general. With ACC synthase induction as a monitor, it is possible to discern and quantify a response 20 to 30 min after addition of elicitor. This early response is close to background levels for 0.1 μg mL^{-1} and almost saturated at 1 μg mL^{-1}. Interpretation of this result as a reflection of elicitor binding leads to the conclusion that the hypothetical receptor displays binding with saturation at about 1 μg mL^{-1}. In most other model systems, elicitor effects are measured much later, for example only after 4 to 6 h in the case of our own ethylene measurements or only after 24 h in the case of most phytoalexin measurements (see ref. 11 for review). Measurements of ethylene biosynthesis after 4 h (Fig. 1) or of ACC synthase activity after 2 h (Fig. 6A) yield a dose-response relationship in which the response increases continuously from 0.1 to 100 μg mL^{-1}, a result difficult to interpret in terms of a simple receptor binding model. The later the responses occur, the more unresolvably intertwined in the response are phenomena like inactivation of elicitors (6), adaptation phenomena, and secondary alterations of metabolism (7). Such late effects, com-

mendable though they may be in bioassays of the elicitor response, are therefore difficult to use in attempts to study and evaluate elicitor perception. Measurements of ACC synthase are well suited for the latter purpose.

CONCLUSION

Our results show that measurements of ethylene production provide a simple and rapid bioassay to monitor the response of plant cells to elicitors. Ethylene production integrates the effects of transient induction of ACC synthase, accumulation of ACC, and the more permanent induction of EFE, yielding smooth dose-response relationships over several orders of magnitude of elicitor concentration. ACC synthase activity itself increases rapidly upon addition of elicitor and decreases equally rapidly upon its withdrawal. Thus, measurements of ACC synthase activity provide a valuable bioassay to monitor the actual state of elicitor perception.

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